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Award Number: W81XWH-09-1-0548

TITLE: Osteoclast Inhibitory Peptide-1 Therapy for Paget's Disease

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REPORT DATE: August 2012

TYPE OF REPORT: Final

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

DISTRIBUTION STATEMENT: Approved for public release;
Distribution unlimited

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

1. REPORT DATE *(DD-MM-YYYY)*	2. REPORT TYPE	3. DATES COVERED *(From - To)*
August 2012	Final	1 August 2009 - 31 July 2012

4. TITLE AND SUBTITLE
Osteoclast Inhibitory Peptide-1 Therapy for Paget's Disease

5a. CONTRACT NUMBER

5b. GRANT NUMBER
W81XWH-09-1-0548

5c. PROGRAM ELEMENT NUMBER

6. AUTHOR(S)
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5d. PROJECT NUMBER

5e. TASK NUMBER

5f. WORK UNIT NUMBER

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
Department of Education, South Carolina
Charleston, South Carolina 29425

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)
U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSOR/MONITOR'S ACRONYM(S)

11. SPONSOR/MONITOR'S REPORT NUMBER(S)

12. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for Public Release; Distribution Unlimited

13. SUPPLEMENTARY NOTES

14. ABSTRACT
Abstract on next page.

15. SUBJECT TERMS
Subject terms on next page.

16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT	18. NUMBER OF PAGES	19a. NAME OF RESPONSIBLE PERSON
a. REPORT	b. ABSTRACT	c. THIS PAGE			USAMRMC
U	U	U	UU	9	19b. TELEPHONE NUMBER *(include area code)*

14. ABSTRACT Paget's disease (PDB) is a focal skeletal disorder which affects approximately 2-3 million people over the age 60 years. PDB is characterized by markedly increased osteoclast formation and excess bone resorption. Both viral and genetic factors have been implicated in the pathogenesis of PDB. Mutations (P392L) in the ubiquitin-associated domain of sequestosome 1 (SQSTM1/p62) gene have been widely identified in PDB patients. We previously detected expression of measles virus nucleocapsid (MVNP) transcripts in osteoclasts from patients with PDB and demonstrated that MVNP expression in normal human osteoclasts precursors induce pagetic phenotype in osteoclasts. Also, we showed targeted over-expression of OIP-1 in the osteoclast lineage inhibits osteoclast formation/bone resorption activity in vivo. Recently, we determined the MVNP and $p62^{P392L}$ regulated gene expression profiling in preosteoclast cells. In this study, we identified MVNP induced CXCL5 expression in preosteoclast cells and that CXCL5 induce RANKL, a critical osteoclatogenic factor expression bone marrow stromal/preosteoblast cells. We demonstrated that OIP-1 inhibit MVNP induced IL-6 expression in bone marrow monocytes. Further, both $p62^{P392L}$ and MVNP induced IL-23 expression and that OIP-1 inhibits IL-23 expression in preosteoclast cells. These studies provide new insights into the molecular mechanisms underlying the high bone turnover in PDB.

15. SUBJECT TERMS

Paget's Disease, measles virus nucleocapsid, sequestosome1, osteoclast, osteoclast inhibitory peptide-1, RANK ligand

Table of Contents

Introduction

Paget's disease (PDB) is a focal skeletal disorder which affects approximately 2-3 million people over the age 60 years in the US. Both viral and genetic factors have been implicated in the pathogenesis of PDB. Mutations (P392L) in the ubiquitin-associated (UBA) domain of sequestosome 1 (SQSTM1/p62) gene have been widely identified in PDB patients (1). Osteoclast is the bone resorbing cell. Paget's disease is characterized by markedly increased osteoclast formation and excess bone resorption. These abnormal osteoclasts contain paramyxo-viral nuclear inclusions and antigens. We previously detected expression of measles virus nucleocapsid (MVNP) transcripts in osteoclasts from patients with PDB. Also, we have shown that MVNP gene expression in normal human osteoclasts precursors results in pagetic phenotype in osteoclasts (2). RANK ligand (RANKL), a critical osteoclast differentiation factor expressed by marrow stromal/preosteoblast cells is increased in PDB (3). We have previously characterized the Ly-6 family member, osteoclast inhibitory peptide-1 (OIP-1/hSca) inhibition of osteoclast formation and bone resorption activity. Furthermore, targeted over-expression of OIP-1 in the osteoclast lineage develops an osteopetrosis bone phenotype in mice due to inhibition of osteoclast formation/bone resorption activity in vivo (4,5). We hypothesize that that OIP-1 inhibition of MVNP and mutant p62(P392L) regulated gene expression abrogate pagetic osteoclast development/bone resorption function. Thus, the proposed studies will facilitate development of novel therapeutic agents to control abnormal osteoclastogenesis and high bone turnover in PDB.

Body:

Task 1. Determine the gene expression profiling with respect to measles virus nucleocapsid (MVNP) and p62 mutant gene expression in pre-osteoclast cells. (Months 1-12): ***Completed***

Task 2. Determine the OIP-1 inhibition of MVNP and p62 mutant regulated gene expression which stimulates osteoclast bone resorption activity. (Months 13-24): ***Completed***

Task 3. Assess the potential of OIP-1 to inhibit MVNP and p62 mutant induced osteotropic cytokines production by osteoclasts and stimulation of RANKL gene expression in bone microenvironment. (Months 25-36):

(a) *Examine the levels of osteotropic cytokines in MVNP and p62 mutant transduced osteoclasts*—In the previous report period, we determined MVNP and $p62^{P392L}$ mutant regulated gene expression profiling during osteoclast differentiation by Agilent microarray (~26,000 genes) analysis. In this report, gene expressionprofile analysis identified MVNP significantly upregulated chemokine ligand-5 (CXCL5) expression. We confirm MVNP transduced normal human bone marrow derived mononuclear cells demonstrated high level mRNA expression of chemokine ligand-5 (CXCL5) (72 fold) (Fig. 1A). Further, real-time PCR analysis of total RNA isolated from bone marrow derived mononuclear cells from patients with PDB demonstrated elevated (~180 fold) levels of CXCL5 mRNA expression compared to normal subjects (n=5) (Fig.1B). We also identified a 5.0 fold increase in serum levels of CXCL5 in patients (n=10) with PDB compared to normal subjects (Fig.1C). In addition, conditioned media obtained from MVNP transduced osteoclast cultures demonstrated increased levels of CXCL5 (Fig. 1D). Further, human bone marrow derived stromal cells were stimulated with CXCL5 (0- 50 ng/ml) for 48 h. Total cell lysates were subjected to Western blot analysis for RANKL expression. Interestingly, CXCL5 significantly increased RANKL expression in dose dependent manner (Fig. 2A). Also, CXCL5 enhances RANKL mRNA expression in these cells (Fig. 2B). In addition, CXCL5 treatment also significantly increased hRANKL gene promoter activity in human bone marrow derived stromal/preosteoblast cell (Fig. 2C). Micro array analysis also identified $p62^{P392L}$ mutant increased the level of cytokine IL-23 (5

fold) mRNA expression in preosteoclast cells. We next examined the effect of IL-23 on RANKL expression in stromal/preosteoblast cells. Human bone marrow derived stromal SAKA-T cells were stimulated with IL-23 (0-50 ng/ml) for 48 h. Total mRNA isolated from these cells were subjected to RT-PCR analysis for RANKL mRNA expression. Interestingly, IL-23 significantly increased RANKL mRNA expression in dose dependent manner (Fig. 3A). Western blot analysis further confirmed that IL-23 significantly increased RANKL expression in these cells (Fig. 3B).

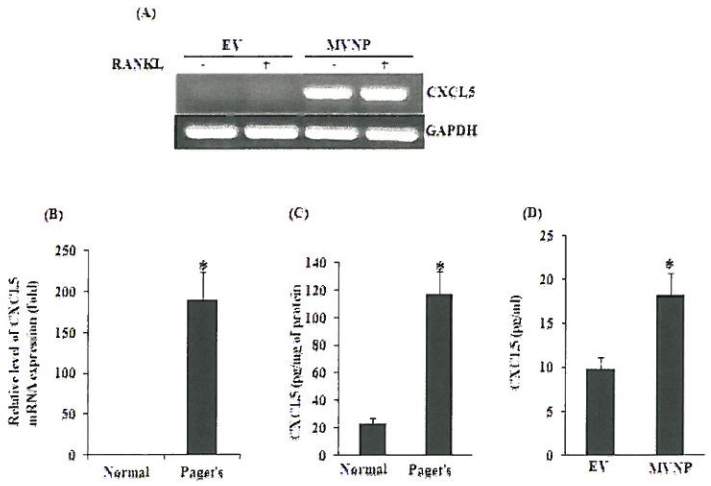

Fig. 1. CXCL5 expression in Paget's disease of bone. (A). Human bone marrow derived monocytes were transduced with control EV or MVNP retrovirus expression vectors and stimulated with RANKL (100 ng/ml) and M-CSF (10 ng/ml) for 48 h. Total RNA isolated was subjected to RT-PCR analysis for CXCL5 mRNA expression. (B). Total RNA isolated from normal and PDB patients (n=5) bone marrow monocytes was subjected to real-time PCR for CXCL5 mRNA expression. (C).CXCL5 levels in serum samples from normal subjects and patients with PDB (n = 10). (D). Human bone marrow cells were transduced with control EV or MVNP retrovirus expression vectors and stimulated with RANKL (100 ng/ml) and M-CSF (10 ng/ml). Serum-free CM was collected for 48 h. The level of CXCL5 was measured by ELISA. Data represent triplicate studies and are shown as mean ± SD. * $p < 0.05$.

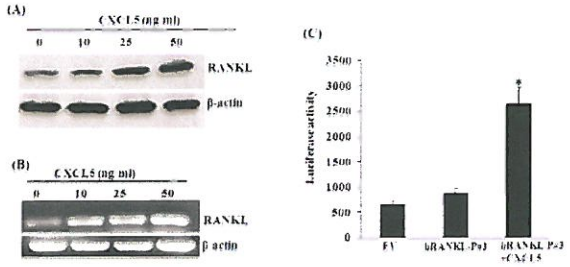

Fig. 2. CXCL5 stimulates RANKL expression in human bone marrow derived stromal/preosteoblast cells. (A) Cells were stimulated with CXCL5 (0-50 ng/ml) for 48 h. Total cell lysates obtained were subjected to Western blot analysis for RANKL expression. (B). Total RNA isolated was subjected to RT-PCR analysis for RANKL mRNA expression. (C). Human bone marrow derived stromal/preosteoblast SAKA-T cells were transfected with hRANKL–Luc reporter plasmid (-2kb). Cells were stimulated with CXCL5 (50ng/ml) for 48 h. Total cell lysates were assayed for luciferase activity. The transfection efficiency was normalized by β-gal activity co-expressed in these cells. Data represent triplicate studies and are shown as mean ± SD. * $p < 0.05$.

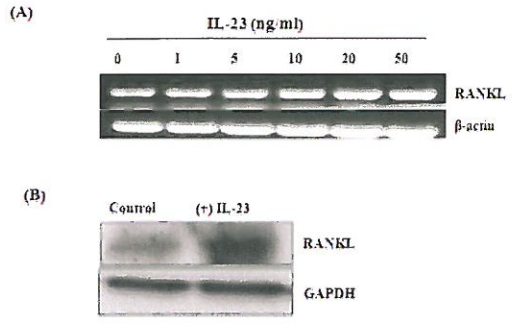

Fig. 3. IL-23 enhances RANKL mRNA expression in human stromal/preosteoblast cells. (A). Human bone marrow derived stromal/preosteoblast cells were stimulated with IL-23 with different concentration (0-50 ng/ml) for 48 h. Total cell RNA isolated was subjected to RT-PCR analysis for RANKL mRNA expression. The mRNA expression was normalized by β-actin amplification in these cells. (B). Human bone marrow derived stromal/preosteoblast SAKA-T cells were stimulated with IL-23 (50 ng/ml) for 48 h. Total cell lysates were subjected to Western blot analysis for RANKL expression. The protein concentration was normalized by β-actin expression in these cells.

(b) *Assess OIP-1 inhibition of MVNP and p62 mutant stimulated osteotropic cytokine expression in pagetic osteoclasts*— Previously, it has been reported that elevated levels of interleukin-6 are associated with PDB and

that IL-6 is an autocrine/paracrine factor which increase osteoclast formation in bone marrow cultures (6). Therefore, we further examined OIP-1 inhibition of IL-6 expression. Human peripheral blood mononuclear cells were transduced with empty vector (EV) and MVNP in presence or absence of OIP-1 c-peptide were stimulated with RANKL for 5 days and condition media was collected, concentrated and IL-6 was measured by ELISA. Consistent with our previous findings, MVNP stimulated osteoclast cells confirmed the increased levels (4.3 fold) of IL-6 when compared to EV transduced osteoclast cells (Fig. 4). We found a significant inhibition of IL-6 in the OIP-1 c-peptide (100 ng/ml) treatment of MVNP transduced cells. These results suggesting that OIP-1 regulate IL-6 mRNA expression in MVNP transduced osteoclast cells. We next used the ELISA to determine secreted levels of IL-6 from *in vitro* cell preparations. Wild-type (WT) and OIP-1 mouse derived bone marrow cells were cultured in the presence of 10 ng/ml M-CSF for 24 h. The non-adherent cells were treated with or without RANKL (100 ng/ml) for 5 days. Condition media collected from these osteoclast cells were measurement for IL-6 levels using ELISA kit according to manufacture protocol. As shown in Fig. 5, cells transduced with MVNP either treated with or without RANKL showed increased IL-6 secretion (5-fold and 8.7 fold) respectively. Condition media from OIP-1 transgenic mice showed a significant decrease in the levels of IL-6 when compared to these cells transduced with MVNP alone and MVNP treated with RANKL.

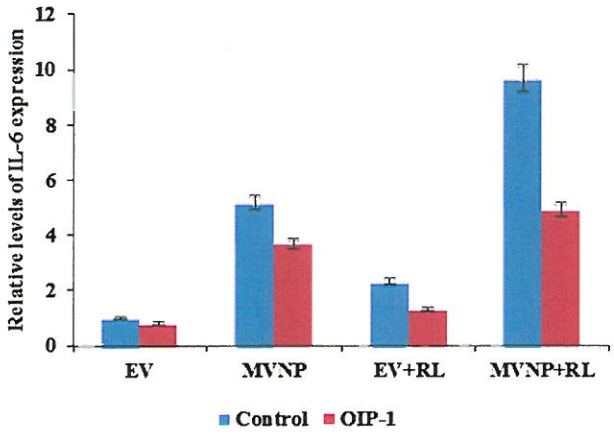

Fig. 4. Wild-type mouse bone marrow derived monocytes (4×10^5/mL) transduced with MVNP or empty vector (EV) were cultured with or without RANKL (100 ng/ml) and treated in presence or absence of OIP-1 c-peptide (100 ng/ml) 48 hours. The cells were treated with or without RANKL (100 ng/ml) for 2 days. Total RNA isolated was subjected to quantitative real-time PCR analysis for IL-6 mRNA expression. The mRNA expression was normalized by β-actin amplification in these cells. Data represent triplicate studies and are shown as mean ± SD. * $p < 0.05$.

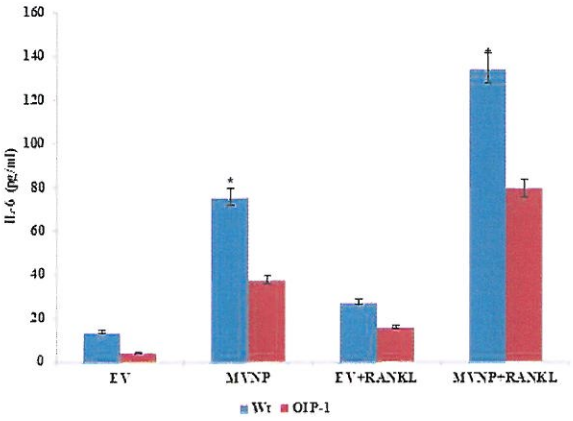

Fig. 5. Wild-type (WT) and OIP-1 mouse derived bone marrow cells were cultured in the presence of 10 ng/ml M-CSF for 24 h. The non-adherent cells were treated with or without RANKL (100 ng/ml) for 5 days. Condition media collected from these osteoclast cells were measurement for IL-6 levels using ELISA kit according to manufacture protocol. Data represent triplicate studies and are shown as mean ± SD. * $p < 0.05$.

(c) *Examine if over-expression of OIP-1 in cells of osteoclast lineage in-vivo suppress elevated levels of RANKL expression in pagetic stroma/preosteoblast cells--* Microarray analysis identified that $p62^{P392L}$ mutant increased (5-fold) IL-23 expression. Studies noted in Task-3b (Fig.3) demonstrated IL-23 significantly

increase RANKL expression. Therefore, we further examined if MVNP induces IL-23 expression and over-expression of OIP-1 in osteoclast lineage inhibit MVNP induced IL-23 expression. As shown in Fig.6, we identified MVNP induced IL-23 expression compared to empty vector (EV) transduced human peripheral blood monocytes cultured to form preosteoclast cells (CFU-GM). Cells transduced with OIP-1 expression vector showed inhibition of MVNP induced IL-23 mRNA expression. In contrast there was no change in the expression levels of IL-23 receptor (IL-23R). Beta actin expression is analyzed to normalize the results.

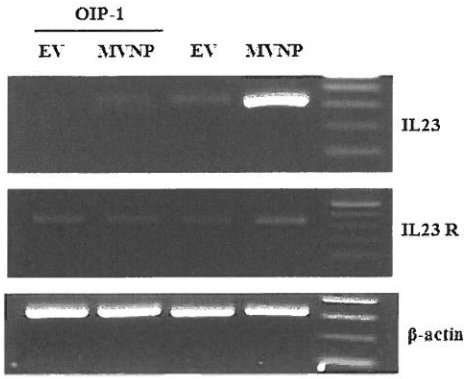

Fig.6. OIP-1 inhibits IL23 expression in MVNP stimulated CFU-GM formed osteoclast precursors. Non-adherent human peripheral blood derived monocytes (4×10^5/mL) transduced with MVNP or empty vector (EV) were cultured with GM-CSF (10 ng/ml) in the presence or absence of OIP-1 in methylcellulose to form CFU-GM colonies. At the end of a 7-day culture period, CFU-GM colonies were collected and total RNA isolated was subjected to quantitative real-time PCR analysis for IL-23 and IL-23 receptor mRNA expression. The mRNA expression was normalized by β-actin amplification in these cells. Data represent triplicate studies and are shown as mean ± SD.

Key Research Accomplishments:

- We identified measles virus nucleocapsid (MVNP) induce CXCL5 chemokine ligand expression in preosteoclast cells. We also identified CXCL5 induce RANKL, a critical osteoclatogenic factor expression bone marrow stromal/preosteoblast cells.
- We demonstrated that OIP-1 inhibit MVNP induced IL-6 expression in bone marrow monocytes. Both $p62^{P392L}$ and MVNP induced IL-23 expression and that OIP-1 inhibits IL-23 expression in preosteoclast cells.

Reportable Outcomes

Article(s): None

Abstract(s):

1. Kumaran Sundaram, D. Sudhaker Rao, William L. Ries and Sakamuri V. Reddy. CXCL5 stimulation of RANK ligand expression in Paget's disease of bone. ASBMR 2012 Annual Meeting, at Minnesota, USA. October 12-15, 2012 (Abstract # SA0421).

Conclusions

In conclusion, we showed MVNP induce the chemokine CXCL5 expression and that CXCL5 modulate RANK ligand expression in bone marrow stromal/preosteoblast cells. Both MVNP and $p62^{P392L}$ mutant enhanced IL-23 levels and that IL-23 induces RANKL expression. Thus, MVNP and p62P392L mutant modulation of cytokine expression regulates RANKL expression and thus provides new insights into the molecular mechanisms underlying the high bone turnover in PDB.

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Appendices: None